Effect of Methyl Jasmonate on Phloem Chemistry of White Ash (*Fraxinus americana*) and Manchurian Ash (*Fraxinus mandshurica*)

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Abstract

The Emerald Ash Borer (EAB; *Agrilus planipennis*) is an exotic invasive species that is killing native ash trees at an alarming rate. This pest has the ability to attack a healthy, native ash tree by bypassing the plant's defenses. White ash (*Fraxinus americana*) is a native species that is of high economic and ecological importance in natural as well as urban environments, but is susceptible to EAB. Manchurian ash (*Fraxinus mandshurica* Rupr.) is native to northeastern Asia and is resistant to attack from EAB. Methyl jasmonate (MeJA) is known to accumulate in angiosperms in response to herbivory and to upregulate host defense responses. We are testing if methyl jasmonate can prime a tree's defenses by inducing accumulation of phloem phenolics, a class of compounds that has been implicated in plant resistance to both pathogens and insects. Half the trees of each species were treated by painting a solution of MeJA on three randomly selected branches to runoff and a water control was applied to the other trees. Sub-treatments of EAB larval homogenate (a surrogate for EAB attack), wound, and non-wound, were applied to one of the three randomly selected braches on all trees. Phenolics were extracted in methanol and the extracts were analyzed by high-performance liquid chromatography. Preliminary results show that fourteen compounds of interest were affected by treatment and that Manchurian ash responded more strongly to MeJA and produced more defense compounds than white ash. These results are discussed in relation to Manchurian ash resistance to EAB.

Introduction

The emerald ash borer (EAB), *Agrilus planipennis* Fairmare, is an exotic invasive species that requires our attention. First identified in July of 2002 outside Detroit Michigan, this invasion has caused the death of tens of millions of ash trees (www.emeraldashborer.info). The United States Department of Agriculture has definitively identified the beetle in the surrounding states of Ohio, Illinois, Indiana, Pennsylvania, West Virginia, Maryland, Missouri, Wisconsin, and Virginia. It has also gone into the Canadian provinces of Ontario and Quebec (www.aphis.usda.gov). The toll of ecological and economic damage continues to grow as it spreads across the country. Ash trees include sixteen native North American species, all susceptible to attack (Poland, 2006), and it is estimated that they account for ten percent of our nation's forests. The unsettling ability of EAB to infest and kill healthy trees in three to five years reveals our native species are defenseless against the exotic borer.

Researchers have had to begin from scratch to understand this pest and develop management techniques. Dr. Sydnor from The Ohio State University has predicted that the EAB could cost Ohio $7.5 billion in the loss of landscape value, and cost of removing and replacing trees (2007). This has spurred a collaborative effort among many researchers, regulatory officials, and the private industry to take to control of the pest. The collaboration has provided a wealth of information on delimiting where the pest is, effective insecticides, potential biological controls, and how it is spreads. One area of ongoing research is the natural defenses of ash trees and improving their ability to combat EAB. A critical element of this research is the Manchurian ash (*Fraxinus mandshurica* Rupr.), a native to northeastern Asia. A common garden study has shown low levels of EAB infestation and associated mortality on Manchurian ash (Rebek et al. 2008). This general resistance is believed to be a result of the coevolutionary history between

the host and pest. Researchers are working to better understand this resistance in attempt to save the North American ash species.

Plants have two forms of defense to protect themselves from attack of pathogens and pests. The first level is innate in the plant. This preformed defense is the first barrier a plant has to an attacker. This includes physical barriers such as the outer bark and trichomes on leaves. It also includes a chemical barrier of constitutive compounds that work to deter pests. These compounds work in various ways such as making the host taste bad to the invader or preventing adequate acquisition of nutrients.

The second form of defense is induced at the time of attack. This inducible defense causes the plant to increase the production of compounds to fight off the invader. The mechanisms behind this defense are similar to animal immunity. Foreign proteins are recognized by the host plant and these receptors initiate a pathway to up regulate compounds to defend against the attack. One class of these compounds is phenolics. These low-molecular weight metabolites can be quickly produced to slow the growth of an invader. Methyl jasmonate, a plant hormone, was found to elicit defense gene expression in this second form of inducible defense in many plant species (Hudgins, 2004). The methylated jasmonic acid is capable of crossover induction effects because the volatile compound can be released and induce neighboring plants (Thaler et al. 2002). "[Methyl Jasmonate] protects the plant from insect infestation and necrotrophic pathogens that kill the host cell before feeding" (Beckers 2006).

It is unknown how methyl jasmonate will affect ash trees and if this compound will make the tree resistant to the EAB. Therefore the objective of this study is to identify differentially expressed phenolics between methyl jasmonate treatment and non-treatment.

Driving Question:

Does methyl jasmonate affect the chemistry of white (*Fraxinus americana*) and Manchurian (*Fraxinus mandshurica*) ash in a way that could make these hosts more resistant to the EAB?

Hypotheses:
1) Methyl jasmonate will prime trees to become potentially more resistant by increasing accumulation of phenolics.

2) Resistance of Manchurian ash to attack from the EAB when compared to native species such as White ash is in part due to differences in host chemistries

Materials and Methods

Twelve white ash (*Fraxinus americana*) cultivar Autumn Purple® and twelve Manchurian ash (*Fraxinus mandshurica*) cultivar Mancana were used in this study. Six trees of each species were treated with methyl jasmonate (MeJA). The remaining twelve trees were treated with the solvent used for MeJA (water). The twelve treated trees received a 100 mM solution of methyl jasmonate one week prior to day zero applied as paint, to runoff. The MeJA-treated trees were separated from the non-MeJA trees by at least 10 m to avoid crossover induction effects. Three branches on each tree were selected as the target sites and receive one of three sub-treatments: 1) unwounded branch (negative control), 2) wounded branch, and 3) wound plus larval homogenate (lyophilized ground EAB larvae re-hydrated in branch with water agar plug). The larval homogenate serves as an artificial challenge of EAB larvae because of the biological restrictions of the pest. Larval attacks can only originate from a egg deposited by a female. Without effective lures, natural attack was not a viable option for this experiment. Larvae collected from infested trees were lyophilized in attempt to maintain the proteins in the homogenate and provide

the effective triggers to artificially challenge the ash trees. The amount of larval homogenate was equivalent to one, fourth instar larva. All treatments were randomized in branches to assure unbiased results.

Sub-treatments 2 and 3 were implemented 5 cm from the stem-branch junction on day zero. On day five, two samples were taken from the unwounded branch, one 5 cm from the stem-branch junction and another 15 centimeters away. The same day, samples were collected from two sites on branches with the wound and wound plus larval homogenate, one from the localized region of the initial wound and the second site was 15 centimeters from the initial wound. These two samples were collected to investigate the local and systemic effects of the sub-treatments. One hundred and forty-four samples were collected and analyzed for changes in phenolic profiles and lignin production related to host defense.

The bark of the sample areas was removed with a razor blade to analyze the phloem. The bark shavings were ground into a fine powder with a mortar and pestle. The tissues were kept below 0° C with liquid nitrogen to preserve the samples. The soluble phenolics were extracted from these samples following a protocol as described in Wallis et al. (2008). 0.1 g fresh ground sample was measured into a 1.5 ml tube with 0.5 mL of MeOH. These tubes were vortexed and allowed to rest at 4° C overnight. The samples were centrifuged at 13,400 rcf for 5 min and the supernatant was transferred into a clean tube. The methanol extraction was repeated once. Combining the supernatants from both extractions provided the samples to be analyzed.

Soluble phenolics were run through a high-performance liquid chromatograph (HPLC). The graphs produced from the HPLC have peaks that correspond to specific soluble phenolic compounds. Peaks showing differing concentrations were targeted. These peaks can then be matched up to peaks already identified in the two ash species. Statistical software was used to

tell if these differences were significant and not caused by random chance. Conclusions were made based on MeJA's ability to change these compounds. Because the larval homogenate sub-treatment show no significant differences from the wound control, the samples were pooled together to provide a larger sample size for statistical analysis. Complete analysis of the sub-treatments was not finished by the end of the project.

The same extracted phenolics were also used to analyze the total soluble phenolics with the Folin-Ciocalteu method discussed in Bonello and Pierce (1993). The extract was diluted tenfold with distilled water and prepared in 1.5 ml tubes with 750 µl of Folin-Ciocalteu reagent. After three minutes, 750 µl of 1M Na_2CO_3 was added to the solution and placed on shaker for one hour. The total phenolics were analyzed in a spectrophotometer at 725 nm wavelength and compared to a standard curve of gallic acid in methanol. Phenolics were expressed as mg/g FW.

The pellets left over from the methanol extraction were used to analyze the lignin content according to the methods of Bonello & Pearce (1993). The pellets were washed in a three part round of water, methanol, and tert-butyl methyl ether to prepare the samples. They were incubated at 86° C for four hours with thioglycolic acid. Lignin was removed from the pellet by two extraction with 1.5 mL of 0.5 M NaOH and precipitated with 300 µl of concentrated HCl at room temperature for four hours. The lignin was measured using a spectrophotometer and compared to a standard curve of lignin: 0, 18, 45, 90, and 180 µg/mL. The data for total phenolics and lignin were analyzed with statistical software to discover differences in the treatments. Data were analyzed by ANOVA with transformations, where appropriate, to meet requirements of normality of residuals and homogeneity of variance. Analyses were conducted using SPSS v. 15 (SPSS Inc. 2007).

Results

Initial analysis of the HPLC chromatographs showed a unique profile for the two ash species with a set number of phenolics eluting for each. The MeJA treatment and sub-treatments offered differing amounts of these phenolics but they were always present. The methyl jasmonate had a clear effect on the phenolic profiles for both species of ash. In figure 1, the chromatographs show fourteen peaks with significant increases resulting from MeJA treatment. These peaks could not be identified by their retention time and UV maxima listed in table 1. None of the peaks were significantly reduced from the treatment. This illustrates MeJA's ability to cause an up regulation of phenolics. The larval homogenate and wound control sub-treatments showed a statistical difference from the negative control. This result was not fully analyzed and no graphs were prepared to illustrate the difference.

The effect of MeJA is further demonstrated when the total soluble phenolics were measured with the Folin-Ciocalteu method. Figure 2 shows that MeJA caused a significant increase by approximately 50% in total phenolic production for both white and Manchurian ash species. Also, the Folin-Ciocalteu method shows the Manchurian ash has significantly more constitutive phenolics than white ash. Further, constitutive lignin concentration was significantly higher in Manchurian than in white ash, but was unaffected from the MeJA treatment (figure 3).

Discussion

Elevated levels of methyl jasmonate in plant tissues, on which insect feeding has been observed, have been correlated with increased resistance to herbivory (Schaller et. al. 2004). Methyl jasmonate induces endogenous resistance mechanisms in plants by up-regulating the production of defensive compounds that are toxic to the invading herbivore (Gatehouse 2002).

The presence of higher levels of lignin as well as total constitutive and induced soluble phenolics in Manchurian ash suggests a role for these compounds in resistance to insect attack, since this species is resistant. An effect of MeJA on individual soluble phenolics was also observed. Therefore, some of these compounds may have critical roles in resistance, since it can be assumed that host responses to herbivore attack can be mimicked by treatment with MeJA. These findings may have applications in advancing our understanding of plant/insect interactions. The results of this study may also inform possible management strategies for controlling pests by exploiting endogenous host resistance mechanisms by priming plants with external elicitors prior to insect attack. Further investigations are needed to test whether these findings correlate with mechanisms of resistance in deciduous trees to wood-borer attack.

There are no previous studies applying methyl jasmonate to the outer bark of ash. It was uncertain if the trees were able to uptake MeJA. This analysis shows a significant difference between the MeJA treatment and the water control. We can conclude that MeJA has an effect on the host chemistry. The sub-treatments were not effective and offer no insight on MeJA's ability to affect resistance to EAB. The ash trees have not responded to the larval homogenate. The proteins of the larvae may have degraded before being applied. The initial result that showed a difference between the non-wound and the wound treatment should be further analyzed to identify where the differences are and if MeJA affected the response.

This work leaves many open doors to further study. Since we were unable to clearly show an effect of MeJA on EAB feeding, a follow up study with another means to examine the potential effect would better answer the driving question. Also, the study was unable to identify the phenolic compounds upregulated by the MeJA treatment. Carefully identifying these compounds and understanding their function will inform us what tools the host plant has to

defend against natural enemies. The future direction in host defense response is to use the identified pathways of defensive compounds and exploit them to quickly respond to current disease and pest pressures.

Conclusions from this learning experience

This experiment proved to be a challenging experience. The entire research process from initial conception through reporting stretched me to think critically and in an unbiased way. I have gained a greater appreciation for each step in the process, seeing first hand the critical importance for an extensive review of past research and a deep understanding of the existing literature for a strong foundation to build a research project. After building the foundation, designing the experiment provided a framework for the project. The developing thoughts and plans on how to carry out the objectives formed the experimental design. The design taught me the importance of replication and randomization. Going into this project I knew both of these were important parts of research, but I had no concept of the extent to which they were carried out. The experiment was a short process of the whole picture. I learned that it is extremely important to great pay attention to detail, in order to have meaningful results. After the experiment, the processing and analyzing of the samples took more time and effort than expected. This portion of the project felt to drag on for an uncomfortably long time. I struggled to understand the different chemical processes undertaken to extract data from the samples. Once the data were obtained, I was lost in analyzing the data to draw meaningful interpretation. The final write-up for the project exposed a critical weakness underlying much of the project. I kept a disordered notebook, lacking vital records, which crippled the project. Of all the lessons

learned throughout the project, the most important has been to keep open communication and foster a strong working relationship between everyone involved.

The immense difficulties of the experimental process and the uncertainty in the results discouraged me from pursuing research further. I began to look for different directions to apply my studies. After a year long internship with the United States Department of Agriculture (USDA), I have seen the critical important of good science in regulatory work. I have resolved to learn from past mistakes and continue to pursue research and advance my education in a plant science graduate program.

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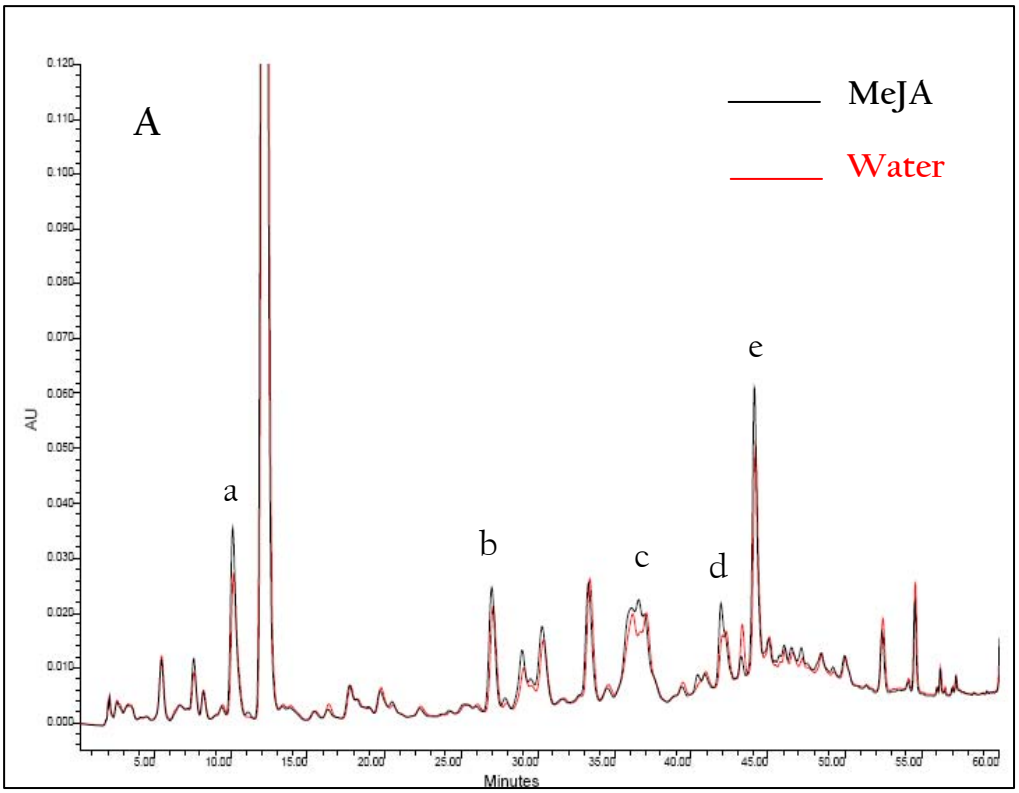

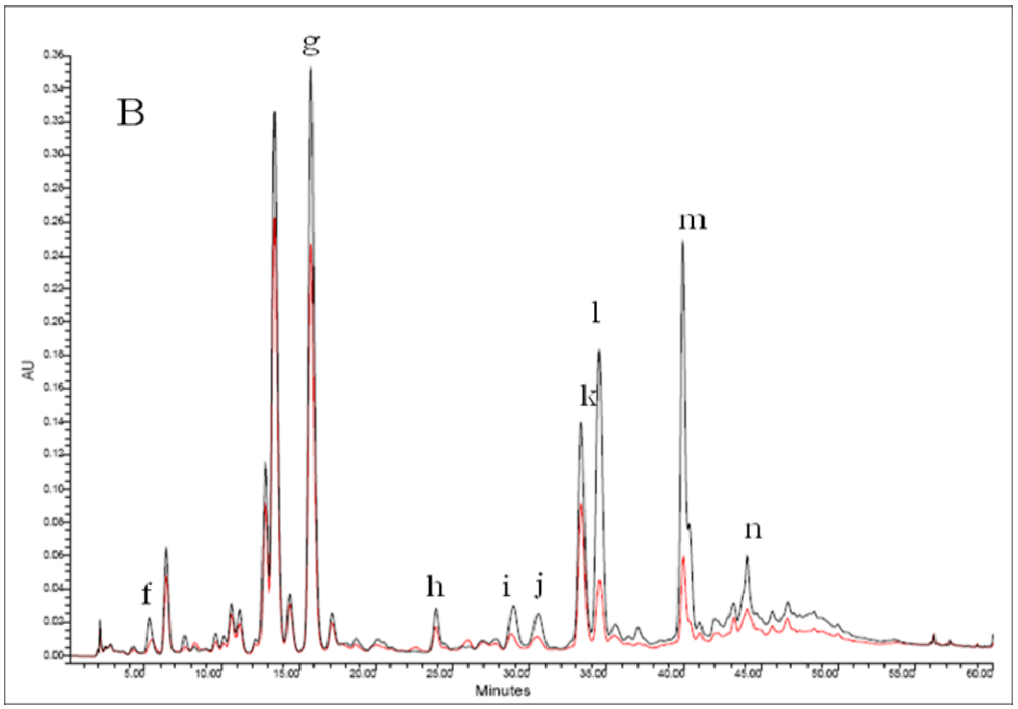

Figure 1. Overlay of HPLC chromatograms comparing constitutive vs. MeJA-induced phenolic profiles of white ash (A) and Manchurian ash (B). Lettered peaks were significantly higher in the MeJA treatment ($P < 0.05$).

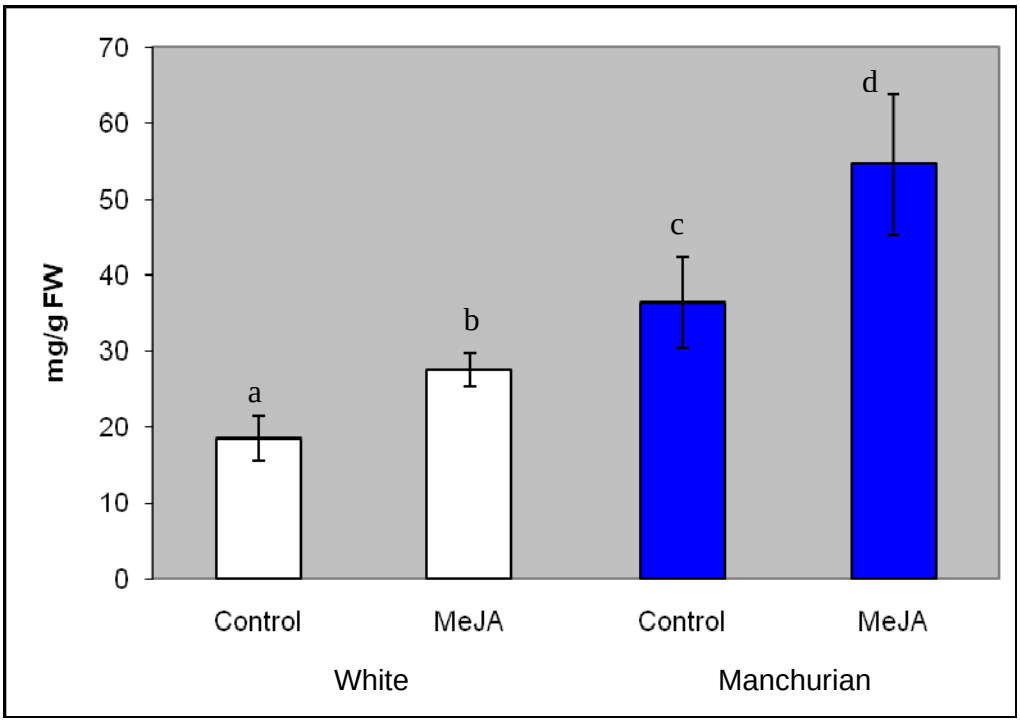

Figure 2. Total soluble phenolics (± SE) from Manchurian and white ash measured using the Folin-Ciocalteau method. Different letters indicate significant differences ($P < 0.05$).

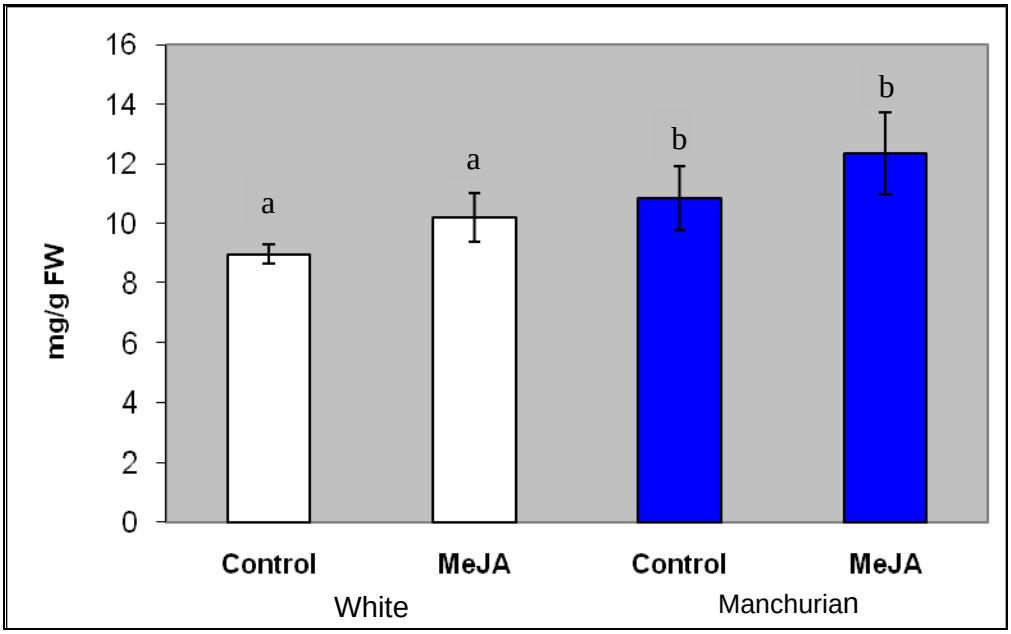

Figure 3. Constitutive and MeJA induced lignin (± SE) in phloem of Manchurian and white ash. Different letters indicate significant differences ($P < 0.05$).

Table 1. Retention time in minutes and UV Maxima for labeled phenolic compounds

Peak ID	Tree species	Retention time (min)	UV Maxima
a	White	10.4	264.7
b	White	27.3	341.9
c	White	33.6	328.7
d	White	42.1	278.9
e	White	44.226	356.2
f	Manchurian	5.463	276.5
g	Manchurian	16.173	290.7, 335.9
h	Manchurian	24.335	sh 300, 341.9
i	Manchurian	29.667	328.7
j	Manchurian	31.0	337.1
k	Manchurian	33.69	278.9
l	Manchurian	35.032	sh 290, 328.7
m	Manchurian	40.243	sh 290, 327.6
n	Manchurian	44.228	237.6, 352.6

sh Shoulder

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The website http://www.emeraldashborer.info is a collaborative effort of the USDA Forest Service, the Michigan Department of Agriculture, the Michigan Department of Natural Resources and USDA Animal and Plant Health Inspection Service(APHIS) as well as Michigan State University, Purdue University and Ohio State University to provide comprehensive, accurate and timely information on the emerald ash borer to the site's visitors. Creation of the site

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http://www.aphis.usda.gov/plant_health/plant_pest_info/emerald_ash_b/background.shtml